AWARD NUMBER: W81XWH-14-1-0470

TITLE: Gestational Exposure as Epigenetic Modifier of Breast Cancer Risk

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CONTRACTING ORGANIZATION: The University of Arizona, Tucson, AZ

REPORT DATE: October 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Annual	30 Sep 2015 - 29 Sep 2016

4. TITLE AND SUBTITLE
Gestational Exposure as Epigenetic Modifier of Breast Cancer Risk

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0470

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER
0010533316-001

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Information from animal models and population studies suggest that mammary tumor promotion in adult life is influenced by prior exposure to carcinogens in early life. The main **purpose** of this project is to investigate whether or not activation of the aromatic hydrocarbon receptor (AhR) induces CpG methylation at the BRCA-1 gene, and if this epigenetic event predisposes to development of triple-negative breast cancers (TNBC). **Major findings**: Preliminary data acquired through the support of this grant indicate that: 1) targeting of the AhR with an AhR antagonist in cell culture experiments with human breast cancer cells harboring hypermethylated BRCA-1 reactivates BRCA-1 and estrogen receptor-α expression; 2) Comparative analyses of human breast tumors indicate the existence of a correlation between higher BRCA-1 promoter methylation and overexpression of AhR in TNBC, but not in luminal type A and B, or Her2-positive breast cancers; and 3) We have developed colonies through breeding of AhR and BRCA-1 conditional mammary tissue knockouts. Pregnant mice from each group have been treated with the AhR agonist TCDD. Mammary tissues from offspring are being collected for analysis of BRCA-1 expression and methylation, and markers of cell proliferation and morphology.
Significance: these data point to overexpression/activation of the AhR to development of a TBNC phenotype characterized by increased BRCA-1 promoter methylation.

15. SUBJECT TERMS
BRCA-1, aromatic hydrocarbon receptor, BRCA-1 promoter methylation, triple-negative breast cancers, estrogen receptor-alpha

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	14	

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1. **INTRODUCTION**

Subject: The BRCA-1 gene encodes a tumor suppressor protein involved in DNA repair and transcription control (1-5). **Purpose**: sporadic breast cancers, which represent the vast majority (~90%) of breast tumor cases, do not have mutations in the BRCA-1 gene (BRCA-$1^{+/+}$), but have absent or markedly reduced levels of BRCA-1 similar to those observed in hereditary BRCA-1 tumors (6-12). **Scope**: understanding the mechanisms that contribute to silencing of BRCA-1 has important implications for the prevention of both hereditary and sporadic breast cancers.

2. **KEYWORDS**

BRCA-1, triple-negative breast cancer, AhR, epigenetic, estrogen receptor-a, hereditary breast cancer, sporadic breast cancer.

3. **ACCOMPLISHMENTS**

- **What were the major goals of the project?**

Goal 1. Investigate interactions between activation of the AhR and BRCA-1 genotype and impact on CpG promoter methylation associated with the TNBC phenotype;

Goal 2. Investigate the combinatorial effects of gestational and postpubertal activation of the AhR on CpG promoter methylation of BRCA-1 and the development of TNBC mammary tumors.

- **What was accomplished under these goals?**

❖ *Accomplishment 1*

One of the main questions raised by this project is whether or not silencing of BRCA-1 is linked to overexpression/activation of AhR-target genes. To answer this question and as a follow-up to data presented in the previous Annual Progress Report, we compared the expression of CYP1A1 and CYP1B1 in UACC-3199 cells in the presence or absence of a-naphthoflavone (αNF), an antagonist of the AhR. We selected these genes because they are direct targets for transcriptional regulation by the AhR. We detected a large increase (~32-fold of control) in *CYP1A1* expression with only modest effects (~1.5-fold increase compared to control) on *CYP1B1* (Fig. 1).

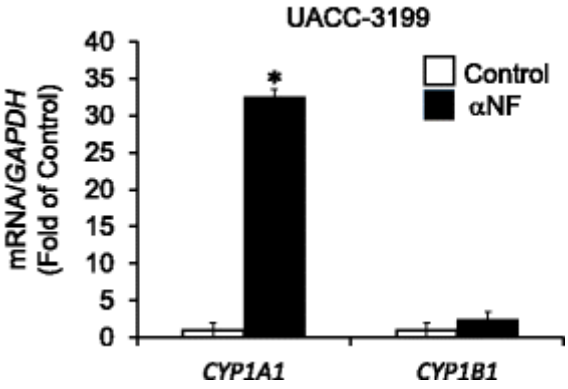

Fig. 1. Bars represent means ± SEM of quantitation of *CYP1A1* and *CYP1B1* mRNA (fold change of control) performed twice in duplicate ($n = 4$) with four repeated measures/sample, and corrected for *GAPDH* mRNA as internal standard. Asterisks represent statistical differences ($P < 0.05$) compared to control.

Then, we compared the effects of αNF on BRCA-1 and ERα expression in MCF-7 and UACC-3199 breast cancer cells. Results illustrated in Fig. 2 confirmed that αNF increased ~2.0- and 3.0-fold of control respectively, BRCA-1 and ERα in UACC-3199 cells, which were however refractory to the treatment with E2 alone or in combination with αNF. On the other hand, as previously reported by our group [13, 14], the treatment of MCF-7 cells with αNF antagonized the E2-dependent induction of BRCA-1. These results were published in 2016 and can be found in reference [15].

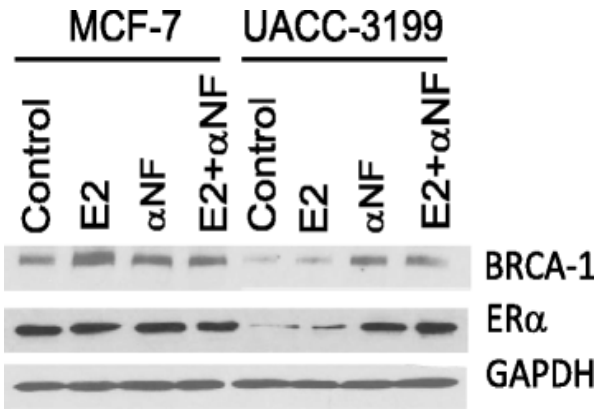

Fig. 2. Differential effects of αNF on BRCA-1 and ERα expression in MCF-7 and UACC-3199 breast cancer cells. Cells were cultured for 72 h in control phenol red-free media (DMEM for MCF-7; RPMI for UACC-31299) supplemented with 10 % charcoal-stripped FCS in the presence or absence of 10 nM E2, alone or in combination with 2 μM αNF. Bands are representative immunocomplexes detected by Western blotting for BRCA-1, ERα, and GAPDH from two independent experiments performed in duplicate.

We measured the impact of AhR targeting on cell proliferation and expression of markers of cell proliferation. The treatment of UACC-3199 cells with αNF (2μM) reduced cell proliferation (60-80%) (Fig. 3A) and cyclin D1 expression (Fig. 3B), regardless of the absence or presence of E2.

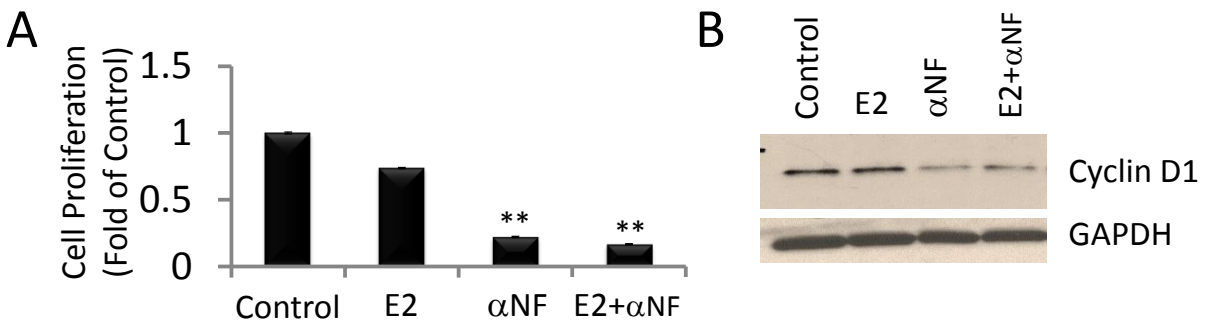

Fig. 3. UACC-3199 cells were cultured for 72 h in control phenol red-free media RPMI supplemented with 10 % charcoal-stripped FCS in the presence or absence of 10 nM E2, alone or in combination with 2 μM αNF. A) Bars represent changes in cells proliferation compared to control. B) Bands are representative immuno-complexes detected by Western blotting for cyclin D1 and GAPDH from two independent experiments performed in duplicate ($n = 4$).

In follow up experiments we found that the upregulation of BRCA-1 protein, and repression of cell proliferation by αNF were paralleled by reduced BRCA-1 promoter CpG methylation, as determined by amplification of bisulfonated DNA obtained from UACC-3199 cells (Fig. 4A), and accumulation of BRCA-1 mRNA (Fig. 4B).

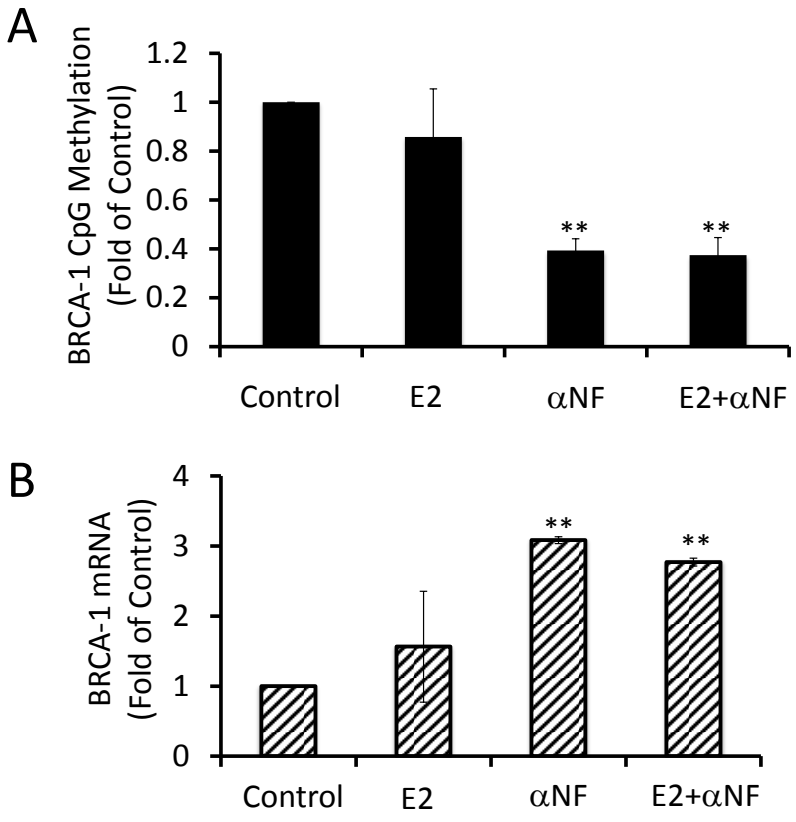

Fig. 4. UACC-3199 cells were cultured for 72 h in control phenol red-free media RPMI supplemented with 10 % charcoal-stripped FCS in the presence or absence of 10 nM E2, alone or in combination with 2 μM αNF. Bars represent changes in BRCA-1 A) CpG methylation and B) mRNA levels compared to control.

Overall, these cell culture studies implied that the effects of αNF, selected as a prototype AhR antagonist, were influenced by cell-context and ERα status, i.e. αNF rescued BRCA-1 and ERα expression in sporadic and ERα-negative UACC-3199 breast cancer cells carrying hypermethylated *BRCA-1*. Conversely, αNF antagonized E2-dependent stimulation of BRCA-1 expression in ERα-positive MCF-7 breast cancer cells. These new data are being compiled for submission and we anticipate their publication by the end of 2016 or early 2017.

❖ *Accomplishment 2*

As a follow-up to the data presented in the previous Annual Progress Report, we have continued the analysis of human tumors with different pathological classification. On average, we observed that *BRCA-1* promoter methylation (M/U ratio) was increased ~6.6-fold in TBNC compared to non-tumor breast tissue (Fig. 5).

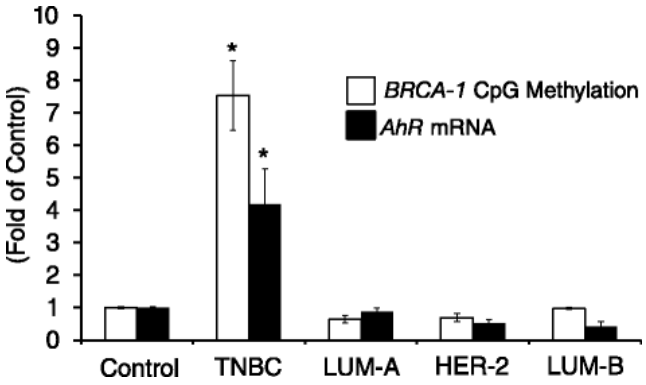

Fig. 5. Human TNBC harbor constitutive *AhR* expression and increased *BRCA-1* promoter CpG methylation. Bars represent quantitation of *BRCA-1* promoter CpG methylation (M/U ratio) and *AhR* expression in human TNBC, LUM-A, LUM-B, and HER-2-positive breast tumors. Asterisks represent statistical differences ($P < 0.05$) compared to non-tumor breast tissue control.

Conversely, compared to non-tumor tissue, there were no differences in the amount of *BRCA-1* promoter methylation in LUM-A, LUM-B, and HER-2-positive breast tumors. Interestingly, the increased *BRCA-1* promoter methylation in TNBC correlated with increased expression (~3.0-fold of control) of *AhR*. Overall, these results denoted that coordinated increase in *AhR* expression and *BRCA-1* gene hypermethylation may be molecular markers of TNBC.

❖ *Accomplishment 3*

Through breeding conducted at the Experimental Mouse Shared Services (EMSS) of the University of Arizona Cancer Center, we have derived the mice colonies to test in vivo how interactions between AhR and BRCA-1 genotype influence mammary tumor development. The colonies are AhR and BRCA-1 conditional Flox knock-out models expressing Cre recombinase in the mammary gland; the groups are as follows:

1. WT BRCA-1 and expressing AhR (BRCA-$1^{+/+}$, $AhR^{+/+}$);
2. WT BRCA-1 and AhR-Flox-CRE knockout (BRCA-$1^{+/+}$, $AhR^{-/-}$);

3. Conditional heterozygous (Flox-CRE) BRCA-1 expressing AhR (BRCA-$1^{+/-}$, $AhR^{+/+}$);
4. Conditional heterozygous (Flox-CRE) BRCA-1 with AhR-Flox-CRE knockout (BRCA-$1^{+/-}$, $AhR^{-/-}$).

Pregnant mice for each genotype were treated at conception day 15 with the AhR agonist TCDD. Female pups from each group were assigned to foster mothers immediately after birth. Mammary tissues from each offspring of the corresponding genotype are being collected at day 50 of age. Mammary tissue are being processed for analyses of morphology; markers of proliferation; and BRCA-1 expression and methylation. During the no-cost extension period of the award, we expect to complete these measurements; analyze and present data at conferences, and submit manuscripts for publication.

Synopsis

Overall, results obtained during this reporting period further support the hypothesis overexpression/activation of the AhR is related to development of breast tumors that are ERα negative and possibly TBNC. We provide evidence that increased expression/activation of AhR induces BRCA-1 promoter methylation. Also, we have gathered new data showing that antagonizing the AhR could provide a new strategy for reactivation of ERa expression, and inhibition of proliferation. In the no-cost extension year of the grant, we will complete analyses of animal tissues with different BRCA-1 and AhR genotype and results are expected to be available for the final report of this award.

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- **What opportunities for training and professional development has the project provided?**

  This project is providing a training opportunity for a new student, Mr. Micah Donovan, who is working on a Master of Science from the University of Arizona. Mr. Donovan is assisting with various aspects of the project including qRT-PCR, Western blot, BRCA-1 methylation, and proliferation analyses. Through these activities he acquired laboratory skills to conduct experiments independently. Also, we advanced our laboratory techniques for the determination of BRCA-1 promoter methylation in human mammary tumors. These techniques will be crucial going forward to testing changes in BRCA-1 promoter methylation.

  Additional opportunities for development included:

- Attendance to Arizona Cancer Center Retreat by Dr. Romagnolo and Dr. Selmin on April 8, 2016.

- Invitation to Dr. Romagnolo to participate in the Metastatic Breast Cancer Working Group, Arizona Cancer Center, The University of Arizona.

- **How were the results disseminated to communities of interest?**

  - Presentation by Dr. Romagnolo of instructional lectures on BRCA-1 and promoter regulation, Nutritional Biology NSc408, Department of Nutritional Sciences.

  - Seminar: Epigenetics of breast cancer – Cancer Biology Interdisciplinary Program – Dec 3, 2015. The University of Arizona.

- **What do you plan to do during the next reporting period to accomplish the goals?**

Mammary tissues from female mice groups with various genetic backgrounds are being harvested and analyzed for BRCA-1/ERα methylation. Also we will perform analyzes of various genes associated with TNBC and AhR, BRCA-1 and ERα expression by RT-PCR and Western blotting.

4. **IMPACT**

- **What was the impact on the development of the principal discipline(s) of the project?**

This project has broad implications for understanding what makes the breast susceptible to cancer. Compounds that activate the AhR include environmental xenobiotics, dietary agents, metabolites of fatty acids, and photoproducts generated in the skin from ultraviolet radiation. Data generated by this project will clarify the impact of specific activation of the AhR and interaction with BRCA-1 genotype on establishment of CpG methylation signatures in particular associated with the development of TNBC, for which prospects for prevention and treatment remain unclear. A possible impact may be the development of clinical trials focused specifically on prevention/treatment of TNBC based on antagonists of the AhR in combination with antiestrogens (i.e. tamoxifen). We are currently engaging discussions with breast cancer surgeons at the University of Arizona Cancer Center on how to develop a future clinical trial.

What was the impact on other disciplines?

- The findings reported so far are likely to make an impact on other disciplines such as those that 1) focus on targeted AhR drug design; 3) nutritional sciences designed for breast cancer prevention based on foods

that possess anti-AhR activities; 3) development of diagnostic tools for predicting the development of TNBC and prevention strategies based on AhR antagonists combined with antiestrogens (i.e. tamoxifen)..

- **What was the impact on technology transfer?** Nothing to report.
- **What was the impact on society beyond science and technology?** Impact beyond the bounds of science, engineering, and the academic world have been:
  - Reaching out to general public and breast cancer interest groups and patients who have expressed interest in the research as well as desire to learn more about the potential applications of our research findings.
  - Based on the knowledge that AhR-activating compounds are present in foods; are generated through uv light exposure of skin; and metabolism of certain dietary fatty acids, the findings presented here have the potential to affect behavior related to sun exposure and food practices as well as increase awareness about the risk of exposure to environmental xenobiotics that activate the AhR (i.e. polycyclic aromatic hydrocarbons, dioxins, etc). As a result of this work, Dr. Romagnolo has been invited to present on the interaction of diet and epigenetic on breast cancer risk. A presentation is scheduled for February 2017 in conjunction with the Research Frontiers in Nutritional Sciences that will be held on the campus of The University of Arizona.

5. **CHANGES/PROBLEMS:** *Nothing to Report.*

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

  - **Journal publications.**

Selmin OI, Daniels KD, Grunwald JT, Ramos SA, Propper CR, Romagnolo DF. 2016. Epigenetics of breast cancer: modifying role of environmental and bioactive food compounds. Mol Nutr Food Res. 60:1310-29.

Romagnolo DF, Papoutsis AJ, Laukaitis C, Selmin OI. Constitutive expression of AhR and BRCA-1 promoter CpG hypermethylation as biomarkers of ERα-negative breast tumorigenesis. BMC Cancer. 2015 Dec 29;15:1026.

  - **Books or other non-periodical, one-time publications.**

Romagnolo DF, Selmin OI. Mediterranean Diet and Prevention of Chronic Diseases. Nutrition Today (Submitted, in revision).

- **Other publications, conference papers, and presentations.**

Conference participation: Nutrients beyond nutrition-5: nutrients before, during and after cancer. 2016. Conference organized by The University La Sapienza in Rome: From primary prevention to therapy and secondary prevention. Octobet 7, 2016. Santa Margherita Ligure, Italy.

- **Website(s) or other Internet site(s)**
  http://uacc.arizona.edu/news/a-perfect-union

  This is a web link to the University of Arizona Cancer Center. It highlights the research supported by this project and makes specific mention of the support received by the Expansion Award.

- **Technologies or techniques.** Nothing to report.

- **Inventions, patent applications, and/or licenses.** Nothing to report.

Other Products. Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Donato F Romagnolo, PhD University of Arizona	PI.
Ornella I. Selmin, PhD University of Arizona	Co-PI
Sueme Darah Lynne University of Arizona	Technician, assisting with breeding protocols and gestational treatments.
Micah Donovan	Graduate Student. Assisted with Western blotting analysis, RNA

	extractions, BRCA-1 promoter methylation from tumor samples.
Andreas Papoutsis, PhD	Postdoc. Assisted with preparation and submission of BRCA-1 manuscript
Christina Laukitis, MD The University of Arizona	Assisted with design of experiments with human breast tumors; organization of tumors based on histo-pathological characteristics;
Tom Doetschman, PhD The University of Arizona	Collaborator on design of experiments with mouse models.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** Nothing to report.
- **What other organizations were involved as partners?** Nothing to report, all personnel was from The university of Arizona.

8. SPECIAL REPORTING REQUIREMENTS

a. **COLLABORATIVE AWARDS:** Nothing to report.

b. **QUAD CHARTS:** Nothing to report.

9. APPENDICES: Nothing to report.